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PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA

ALESSANDRO FATICA,¹ DAVID TOLLERVEY,² and MENSUR DLAKIĆ³

¹Department of Genetics and Molecular Biology, University of Rome "La Sapienza," Rome 00185, Italy

²Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland

³Department of Microbiology, Montana State University, Bozeman, Montana 59717, USA

ABSTRACT

Nob1p (Yor056c) is essential for processing of the 20S pre-rRNA to the mature 18S rRNA. It is part of a pre-40S ribosomal particle that is transported to the cytoplasm and subsequently cleaved at the 3' end of mature 18S rRNA (D-site). Nob1p is also reported to participate in proteasome biogenesis, and it was therefore unclear whether its primary activity is in ribosome synthesis. In this work, we describe a homology model of the PIN domain of Nob1p, which structurally mimics Mg²⁺-dependent exonucleases despite negligible similarity in primary sequence. Insights gained from this model were used to design a point mutation that was predicted to abolish the postulated enzymatic activity. Cells expressing Nob1p with this mutation failed to cleave the 20S pre-rRNA. This supports both the significance of the structural model and the idea that Nob1p is the long-sought D-site endonuclease.

Keywords: rRNA processing; ribosome synthesis; endonuclease; homology modeling

INTRODUCTION

The synthesis of ribosomes is a major metabolic pathway in all cells. To assist in the processing of rRNA and its packaging with ribosomal proteins, more than 150 *trans*-acting proteins are recruited. Many or all of the major intermediates in the processing of the rRNA precursors have been identified (for review, see Kressler et al. 1999; Venema and Tollervey 1999), and an increasingly detailed picture of the order of events in ribosome assembly is emerging (Dragon et al. 2002; Fatica et al. 2002, 2003b; Grandi et al. 2002; Peng et al. 2003; Saveanu et al. 2003; Schafer et al. 2003; Jakovljevic et al. 2004; Krogan et al. 2004). In addition, recent studies have shed light on the mechanisms governing the export of ribosomal subunits to the cytoplasm (Ho et al. 2000; Bassler et al. 2001; Gadal et al. 2001; Milkereit et al. 2001; Moy and Silver 2002; Fatica et al. 2003b; Kallstrom et al. 2003; Dlakic and Tollervey 2004; Oeffinger et al. 2004). However, a notable gap in our understanding of pre-rRNA

processing is the identity of nucleases responsible for several of the cleavage steps (for review, see Fatica and Tollervey 2002).

Nob1p copurifies with late, cytoplasmic 40S pre-ribosomal particles (Schafer et al. 2003) and is essential for cleavage of site D in the 20S pre-rRNA (Fatica et al. 2003a). The C-terminal tail of ribosomal protein rpS14 is also required for this final step in 18S rRNA maturation (Jakovljevic et al. 2004). In addition, Nob1p was implicated in proteasome biogenesis (Tone and Toh-e 2002), raising the possibility that its role in 20S pre-rRNA processing might be indirect or secondary. Nob1p contains a predicted PIN domain, and the structure of an archaeal PIN domain was recently solved and shown to have structural homology to T4 phage RNase H and flap endonucleases (Arcus et al. 2004). This confirmed an insightful suggestion that PIN domains function as nucleases in nonsense-mediated mRNA decay and RNAi, which was based on scant sequence homology to Mg²⁺-dependent exonucleases (Clissold and Ponting 2000). Together, these results suggested that Nob1p might be the endonuclease that cleaves site D. We used the archaeal PIN structure to build a three-dimensional model of the PIN domain of Nob1p and designed point mutations in conserved acidic residues (shown in Fig. 2 of Fatica et al. 2003a). These experiments support the model that Nob1p is

Reprint requests to: Mensur Dlakic; Department of Microbiology, 109 Lewis Hall, Montana State University, Bozeman, Montana 59717, USA; e-mail: mdlakic@montana.edu; fax: (406) 994-4926.

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the nuclease responsible for cleavage of the 20S pre-rRNA at site D.

RESULTS AND DISCUSSION

PIN domains are ~100 residues long and are found in all three kingdoms of life (Makarova et al. 1999; Clissold and Ponting 2000). The structure of a representative PIN domain from an archaeal genome revealed a fold similar to exonucleases (Arcus et al. 2004). In particular, the PIN domain displayed sequence conservation and spatial clustering of metal-chelating acidic residues that is similar to T4 phage RNase H and flap endonucleases, and it is presently classified in the same fold family as a group of 5' to 3' exonucleases (Murzin et al. 1995). This domain is organized as a tetramer that forms a ring structure with a central hole that could accommodate ssDNA or RNA, but not dsDNA. In vitro experiments confirmed that the enzyme cleaves long 5' to 3' single-stranded DNA overhangs in Mg^{2+} -dependent manner (Arcus et al. 2004). We used this structure as a template for a homology model of the Nob1p PIN domain. Four acidic residues were previously identified as potentially important based on primary sequence conservation among Nob1p orthologs (see Fig. 2 in Fatica et al. 2003a), and the model shows that these cluster together within the putative active site (residues are shown in ball-and-stick representation in Fig. 1A). We also assembled an in silico tetramer (Fig. 1B) with subunit arrangements identical to that of archaeal PIN domains (Arcus et al. 2004) to assess whether

the remainder of Nob1p, positioned at the C terminus of the PIN domain, would prevent tetramer formation. Because C-termini of PIN domains point outside of the ring structure (indicated with arrows in Fig. 1B), the rest of the protein could viably fold on the outside of the ring. It should be noted, however, that there are currently no data on the oligomeric state of Nob1p, and this ring-shaped structure should be regarded only as one possible spatial arrangement.

The model in Figure 1A is colored according to evolutionary conservation among Nob1p homologs (Pei and Grishin 2001), with the most conserved residues in red and the least conserved in blue, giving an indication of their relative functional importance. Residues D15, E43, and D92 in Nob1p show greater conservation than residue D110. Based on this result, we constructed mutants carrying substitutions in two aspartate residues, D92N and D110N. Substitutions of acidic residues with their amides are predicted to cause minimal changes in physicochemical properties of the protein other than its charge, yet they are sufficient to abolish coordination of metal ions within the active sites of nucleases (Baker and Luo 1994). We anticipated that the D92N mutation was more likely to inhibit pre-rRNA cleavage, since D92 is absolutely conserved in all PIN-domain proteins. To assess the ability of the mutant forms of Nob1p to support growth, they were expressed constitutively from a plasmid in a strain in which the endogenous *NOB1* gene was under the control of a repressible *GAL* promoter (strain *GAL::HA-nob1* described in Fatica et al.

2003a). Serial dilutions of transformed strains were plated on medium containing either galactose or glucose, and their growth was analyzed. As shown in Figure 2A, the conditional *GAL::HA-nob1* strain transformed either with an empty vector or the Nob1p^{D92N} mutant failed to grow on glucose, while strains complemented by plasmids expressing wild-type Nob1p or the Nob1p^{D110N} mutant grew. These results confirm that the residue D92 is required for Nob1p function. In contrast, the less-conserved residue D110, although also positioned within the active site, is not essential for function.

Considering the proposed role of Nob1p in proteasome maturation (Tone and Toh-e 2002), it remained formally possible that the D92N mutation affected some cellular process other than rRNA processing. To test whether the growth defects correlate with the inhibition of pre-rRNA cleavage, we performed Northern analyses of high molecular-weight RNA species from strains

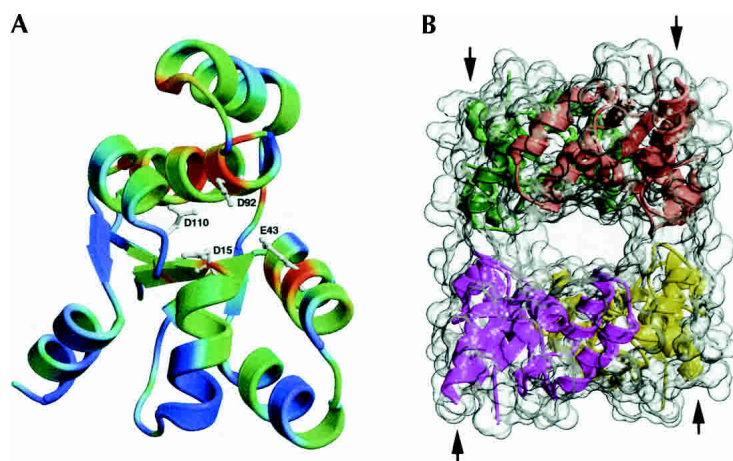


FIGURE 1. Three-dimensional model of the PIN domain of Nob1p. (A) The model was built using MODELLER (Sali and Blundell 1993). It has good stereochemistry and passes the quality criteria for homology models (Sanchez and Sali 1998). The model is colored by positional sequence conservation among eukaryotic and archaeal homologs of Nob1p (Pei and Grishin 2001). Red color indicates the most conserved residues and blue stands for the least conserved ones. The colors in between correspond to intermediate levels of conservation. Four acidic residues clustered in the putative active site are shown in ball-and-stick representation. (B) Tetrameric assembly of Nob1p PIN domains based on the similar arrangement of archaeal PIN domains (Arcus et al. 2004). The transparent molecular surface shows the real dimensions of the central hole that could accommodate RNA or ssDNA but not dsDNA. Arrows indicate positions of the C-termini.

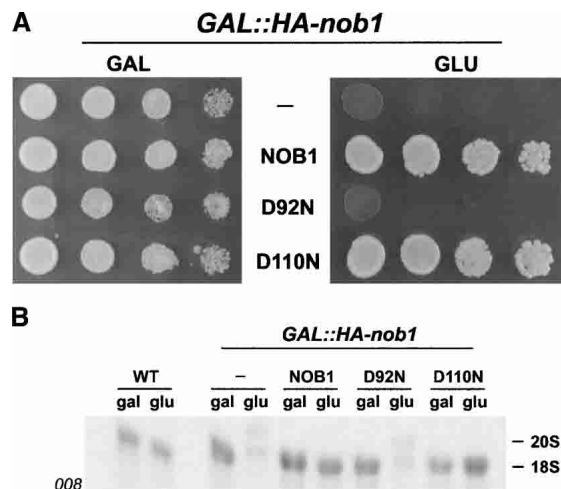


FIGURE 2. Mutation in the PIN domain affects the growth rate (A) and 20S cleavage (B). Growth and handling of *S. cerevisiae* were by standard techniques. The wild-type *NOB1* and D92N and D110N mutants were inserted into the EcoRI–XhoI sites of plasmid p413ADH (Mumberg et al. 1995) to make constructs ADH-*NOB1*, ADH-D92N, and ADH-D110N. Yeast strain YAF34 (Fatica et al. 2003a) was transformed with the empty vector (lane –), ADH-*NOB1* (lane *NOB1*), ADH-D92N (lane D92N), and ADH-D110N (lane D110N). Serial dilutions were grown in the appropriate selective medium containing galactose or glucose at 30°C (A). RNA was extracted from the strains described above 12 h after the glucose shift and assayed by Northern analysis (B) using an oligonucleotide 008 specific for 18S rRNA (Fatica et al. 2003a).

described above. Following transfer to glucose medium, the *GAL::HA-nob1* strain showed a greatly reduced level of 18S rRNA and accumulation of 20S pre-rRNA (Fig. 2B). The relatively low steady-state levels of 20S pre-rRNA seen in the *Nob1p*-depleted strain in Figure 2B reflect the instability of this species, which was clearly accumulated when assayed by pulse-chase labeling in vivo (see Fig. 5A in Fatica et al. 2003a). Complementation with a plasmid expressing wild-type *Nob1p* or the *Nob1p*^{D110N} mutant restored 20S processing. In contrast, the plasmid expressing the *Nob1p*^{D92N} mutant did not support 20S cleavage, linking this point mutation directly to rRNA processing.

A recent study presented data for the involvement of *Nob1p* and *Pno1p* (Yor145c) in proteasome function (Tone and Toh-e 2002). These two proteins, however, have no domains known to be associated with proteasome function, but do have domains associated with RNA metabolism, for example, the PIN domain in *Nob1p* and the KH-domain in *Pno1p* (Peng et al. 2003; Senapin et al. 2003; Vanrobays et al. 2004). In light of our earlier work (Fatica et al. 2003a) and the data presented here, it seems clear that both *Nob1p* and *Pno1p* (also called *Rp20p* or *Dim2p*) have primary functions in ribosome synthesis. This conclusion does not exclude indirect roles for *Nob1p* and *Pno1p* in proteasome formation. It has been proposed that RNA processing and degradation are linked with proteasome functions (Koonin et al. 2001), and several *trans*-acting ribosomal factors are

involved in multiple cellular processes (Du and Stillman 2002; Oeffinger et al. 2002; Oeffinger and Tollervey 2003).

The work presented here shows that a PIN domain is required for pre-rRNA cleavage in addition to previously proposed roles in RNAi and nonsense-mediated mRNA decay (Clissold and Ponting 2000). This recruitment of PIN domains for pre-rRNA processing has, to date, been found only in Archaea and Eukaryotes. Studies are underway to assess the roles of other PIN-domain proteins of *Saccharomyces cerevisiae* in RNA processing.

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